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Proton Motive Force-Driven and ATP-Dependent Drug Extrusion Systems in Multidrug-Resistant *Lactococcus lactis*

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Three mutants of *Lactococcus lactis* subsp. *lactis* MG1363, termed Eth^R, Dau^R, and Rho^R, were selected for resistance to high concentrations of ethidium bromide, daunomycin, and rhodamine 6G, respectively. These mutants were found to be cross resistant to a number of structurally and functionally unrelated drugs, among which were typical substrates of the mammalian multidrug transporter (P-glycoprotein) such as daunomycin, quinine, actinomycin D, gramicidin D, and rhodamine 6G. The three multidrug-resistant strains showed an increased rate of energy-dependent ethidium and daunomycin efflux compared with that of the wild-type strain. This suggests that resistance to these toxic compounds is at least partly due to active efflux. Efflux of ethidium from the Eth^R strain could occur against a 37-fold inwardly directed concentration gradient. In all strains, ethidium efflux was inhibited by reserpine, a well-known inhibitor of P-glycoprotein. Ionophores which selectively dissipate the membrane potential or the pH gradient across the membrane inhibited ethidium and daunomycin efflux in the wild-type strain, corresponding with a proton motive force-driven efflux system. The ethidium efflux system in the Eth^R strain, on the other hand, was inhibited by *ortho*-vanadate and not upon dissipation of the proton motive force, which suggests the involvement of ATP in the energization of transport. The partial inhibition of ethidium efflux by *ortho*-vanadate and nigericin in the Dau^R and Rho^R strains suggest that a proton motive force-dependent and an ATP-dependent system are expressed simultaneously. This is the first report of an ATP-dependent transport system in prokaryotes which confers multidrug resistance to the organism.

Multidrug resistance (MDR) is the intrinsic or acquired resistance of cells to various structurally and functionally unrelated toxic compounds. For various mammalian cells, it has been established that MDR is the result of active extrusion of drugs from the cells, a process that is catalyzed by an ATP-dependent transport protein termed P-glycoprotein or MDR1 (14). P-glycoprotein confers resistance to vinca alkaloids, anthracyclines, actinomycin D, valinomycin, gramicidin D, and phosphonium ions (4, 7, 9). P-glycoprotein is classified among members of the ATP-binding cassette (ABC) proteins (15) or traffic ATPases (26), to which belong prokaryotic and eukaryotic transport systems that facilitate either uptake or extrusion of substrates. Because of the importance of MDR in the failure of drug-based treatment of cancers and parasital infections, most attention has been focused on eukaryotic MDR systems. Recently, however, several MDR-like systems in both gram-positive (31, 42) and gram-negative (11, 20, 23) bacteria as well as in archaea (27) have been described. The occurrence of acquired resistance in bacteria, and especially in pathogenic bacteria like enterococci, staphylococci (35), and *Mycobacterium tuberculosis* (3), also poses serious problems to public health. MDR in these organisms is believed to be the result of active extrusion by broad-specificity transport systems.

Resistance of bacteria to the ethidium ion has been known for a long time (16). Ethidium readily crosses the membrane by passive diffusion and intercalates between the bases of polynucleotides, thereby exerting its growth-inhibitory and mutagenic effects. The presently characterized bacterial ethidium resistance mechanisms are all membrane-located extrusion systems. They can be divided into two homology groups. To the first group belong several tetracycline and other antibiotic resistance efflux systems, multidrug efflux systems, and sugar uptake systems (2, 37). Recently, a mammalian vesicular amine transporter was added to this group (22). These proteins have either 12 or 14 putative membrane-spanning helices, and a few of these carriers have been shown to catalyze secondary transport (reviewed in references 21 and 32). The second group includes the products of highly similar MDR genes from various *Staphylococcus* species and a methyl viologen resistance determinant from *Escherichia coli* (8). The polypeptides of this group have only four putative membrane-spanning helices and are probably also proton gradient-driven extrusion systems. The ethidium efflux systems of both homology groups have different specificities, but all confer cross-resistance to or catalyze efflux of unrelated compounds, like acriflavine, phosphonium ions, quaternary ammonium ions, aminoglycosides, rhodamine 6G, and chloramphenicol (8, 25, 31, 39).

In this paper we describe the isolation of three MDR mutants of *Lactococcus lactis*. Characterization of the strains indicates that transport of ethidium and daunomycin in *L. lactis* can be mediated by two different systems that differ in their mode of energization, i.e., a proton motive force (Δp)- and an ATP-dependent system.

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MATERIALS AND METHODS

Growth and preparation of organisms. *L. lactis* MG1363 was grown at 30°C in medium A, which contained (per liter) 10 g of tryptone, 10 g of Lab-Lemco powder (Oxoid, Basingstoke,

England), 5 g of yeast extract, 2 g of K_2HPO_4, 0.2 g of $MgSO_4$, and 35 mg of $MnSO_4$, adjusted to pH 6.4 with HCl. Sterile 25 mM glucose or 25 mM galactose plus 10 mM L-arginine was added separately.

For experiments with washed cell suspensions, exponentially growing cultures were harvested and washed three times in 50 mM potassium phosphate, pH 7.0, supplemented with 5 mM $MgSO_4$. For inhibitor studies with *ortho*-vanadate, potassium phosphate was replaced by potassium-HEPES [4-(2-hydroxyethyl)-1-piperazinethane-sulfonate] at equimolar concentrations. When necessary, cells were de-energized by incubation for 30 min with 10 mM 2-deoxyglucose at 30°C followed by three washing steps in the appropriate buffer.

Selection of drug-resistant mutants. Mutants of *L. lactis* were isolated by selecting for growth in medium supplemented with the toxic compound. Small amounts of exponentially growing cultures were transferred to medium containing an increased concentration of the toxic compound, after which this step was repeated with stepwise increasing concentrations. The final concentrations used were 200 μM ethidium bromide, 200 μM daunomycin, or 100 μM rhodamine 6G, and the corresponding mutants were termed Eth^R, Dau^R, and Rho^R, respectively.

Determination of growth rate. The growth rate was determined in sterile low-protein-binding microplates (Greiner, Frickenhausen, Germany). Aliquots of 250 μl of medium A with various concentrations of different drugs were inoculated with exponential growing cultures of the wild-type and mutant strains of *L. lactis*. Growth was monitored by measuring the A_{690} every 10 min in a multiscan photometer (Titertek Multiskan MCC/340 MKII; Flow Laboratories, Lugano, Switzerland). Growth rates were determined by nonlinear least-squares fitting of the absorbance data to an equation describing bacterial growth (43).

Ethidium efflux. (i) A washed and de-energized cell suspension (0.2 mg of protein per ml) was incubated at 30°C with 5 μM ethidium bromide and after 2 h was energized with glucose (25 mM). At various times 0.75-ml samples were centrifuged for 3 min at maximal speed (15,000 × *g*) in an Eppendorf centrifuge. Supernatant (0.5 ml) was carefully pipetted off and diluted into 1.5 ml of buffer containing a 0.1 mM concentration of base pairs of fragmented calf thymus DNA (prepared as described below). The fluorescence of this solution was measured with excitation and emission wavelengths of 500 and 580 nm, respectively, using slit widths of 5 and 10 nm, respectively. Fluorescence was measured with a Perkin-Elmer LS 50B fluorometer with computer-controlled data acquisition and storage. A calibration curve with ethidium concentrations in the range of 0 to 10 μM was found to be linear and was used to estimate the concentration in the supernatant. (ii) Ethidium efflux was also estimated from the fluorescence of the ethidium-polynucleotide complex in the cell. Ethidium displays approximately a 10-fold increase in fluorescence quantum yield upon binding to DNA or RNA (19). This property was used to monitor indirectly the intracellular ethidium concentration (18, 24). A washed cell suspension (0.2 mg of protein per ml) was incubated for 10 min with 10 μM ethidium bromide at 30°C to allow the ethidium to diffuse across the membrane. Ethidium efflux was initiated by the addition of glucose (25 mM). Fluorescence was integrated every 0.5 s with excitation and emission wavelengths of 500 and 580 nm, respectively. No corrections were made for light scatter or absorption by cells, since these parameters varied only slightly during the course of an experiment.

Free internal ethidium concentration. The free intracellular ethidium concentration was estimated from the ethidium fluorescence in cells, assuming rapid equilibration between ethidium bound to polynucleotides and the internal pool of free ethidium (40). Cell suspensions were de-energized with 10 mM 2-deoxyglucose, and the transmembrane ion gradients were dissipated with nigericin (1 μM) plus valinomycin (1 μM) in order to prevent accumulation or efflux. Subsequently, the cells were diluted to 0.2 mg of cell protein per ml in 50 mM potassium-HEPES–5 mM $MgSO_4$ (pH 7.0), containing various concentrations of ethidium bromide (0.25 to 20 μM). The total fluorescence (F_{TOT}) of the cell suspension with ethidium is the sum of the background fluorescence due to light scatter and/or absorption by cells (F_0), the intrinsic ethidium fluorescence (F_{ETH}), and the enhanced fluorescence of ethidium due to the binding to polynucleotides (F_{BOUND}). F_{BOUND} can then be calculated according the equation $F_{BOUND} = F_{TOT} - F_{ETH} - F_0$. Within certain limits, F_{BOUND} is linearly related to $[Eth^{BOUND}]$ with a fluorescence constant $K_{F\text{-}BOUND}$. Binding of ethidium to polynucleotides and the resulting fluorescence can be described by a simple Scatchard-type binding model in which

$$[Eth^{BOUND}] = \frac{F_{BOUND}}{K_{F\text{-}BOUND}} = \frac{\frac{F_{BOUND\text{-}MAX}}{K_{F\text{-}BOUND}} \cdot [Eth^{FREE}]_{IN}}{K_D + [Eth^{FREE}]_{IN}} \quad (1)$$

where $F_{BOUND\text{-}MAX}$ represents the fluorescence enhancement from the maximal amount of ethidium bound to polynucleotides and K_D is the dissociation constant. F_{BOUND} was calibrated as a function of the free external ethidium concentration ($[Eth^{FREE}]_{OUT}$) at steady state, assuming that $[Eth^{FREE}]_{OUT} = [Eth^{FREE}]_{IN}$. $[Eth^{FREE}]_{OUT}$ was measured in the supernatant fraction as described under "Ethidium eflux" above. K_D and $F_{BOUND\text{-}MAX}$ were determined by nonlinear least-squares fitting of equation 1 to the calibration data. Rearranging equation 1 gives

$$[Eth^{FREE}]_{IN} = \frac{F_{BOUND} \cdot K_D}{F_{BOUND\text{-}MAX} - F_{BOUND}} \quad (2)$$

From equation 2 and the fitted values for $F_{BOUND\text{-}MAX}$ and K_D, $[Eth^{FREE}]_{IN}$ can be calculated from the observed fluorescence (F_{BOUND}). For the calculations a specific internal volume of 3.6 μl/mg of cell protein was used (33).

Preparation of fragmented calf thymus DNA. Calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) was dissolved in water and sonicated 10 times for 15 s each with an MSE Soniprep probe-sonicator at an amplitude of 6 μm with the tube submerged in ice-water (6). The solution was filtered through an Amicon cellulose nitrate filter with a pore size of 0.2 μm. The base pair concentration was calculated from the A_{260}, using an extinction coefficient of 13.3 $mM^{-1} \cdot cm^{-1}$.

Materials. Ethidium bromide was obtained from Merck, Darmstadt, Germany. Daunomycin (daunorubicin) and reserpine were obtained from Sigma Chemical Co. All other chemicals were reagent grade and obtained from commercial sources.

RESULTS

The MDR phenotype of *L. lactis* mutants. Three *L. lactis* MG1363 mutants, termed Eth^R, Dau^R, and Rho^R, were isolated from liquid cultures growing at 200 μM ethidium bromide or daunomycin or at 100 μM rhodamine 6G, respectively. Cross-resistance was tested for a number of toxic compounds whose toxicities were established on agar plates. A wild-type

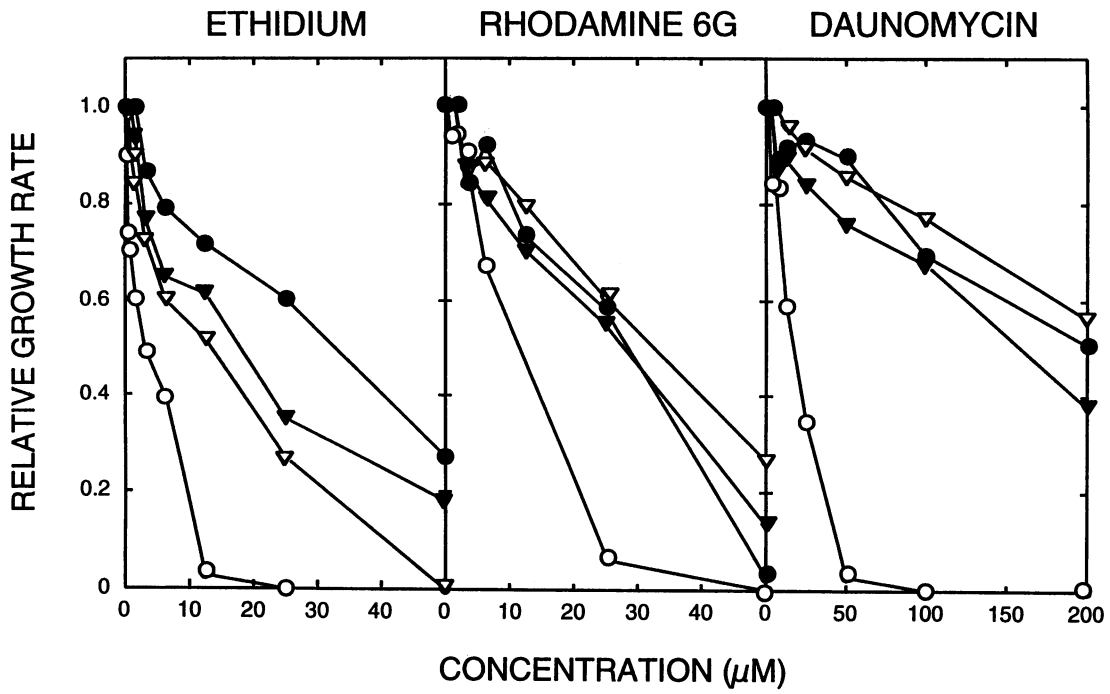

FIG. 1. Cross-resistance of *L. lactis* mutants resistant to ethidium bromide, daunomycin, and rhodamine 6G. *L. lactis* subsp. *lactis* MG1363 (wild type) (○) and the mutants Eth^R (●), Dau^R (▽), and Rho^R (▼) were grown on medium A in the presence of different concentrations of ethidium, rhodamine 6G, or daunomycin. The relative growth rate is plotted as a function of the drug concentration. The actual growth rates of the various strains with no drug varied between 0.9 and 1.1 h^{-1}.

culture was confluently plated, and filter discs containing a high concentration of a toxic compound were placed on top of the agar. Compounds which caused large halos, indicative of growth inhibition, were selected for further analysis. Results with three compounds that caused a clear difference in growth rate between the wild-type strain and the isolated mutants are shown (Fig. 1). The mutants were cross resistant to ethidium, daunomycin, and rhodamine 6G but also to a number of other unrelated drugs, including gramicidin D, actinomycin D, and quinine (data not shown). In fact, irrespective of the selective agent, the mutants were resistant to a similar set of toxic compounds, which is a clear indication for an MDR phenotype in *L. lactis*.

Ethidium efflux from *L. lactis* cells. Ethidium efflux was assayed directly by measuring the ethidium concentration in the medium during the course of a transport experiment. The internal ethidium concentration has been calculated from the difference between the concentrations of total and external ethidium. Ethidium efflux was observed upon energization of *L. lactis* wild-type strain MG1363 and Eth^R (Fig. 2). The initial efflux rates, which were determined by taking the slope (tangent) of the line fitting the initial fluorescence decrease, is approximately fivefold higher for Eth^R than for the wild-type strain. Ethidium efflux was also measured indirectly by monitoring the fluorescence of the intracellular ethidium-polynucleotide complex. The fluorescence intensity after addition of ethidium and before addition of the energy source was normalized to 100%. This fluorescence reflects the transport of ethidium across the cytoplasmic membrane and the rapid binding equilibrium of intracellular ethidium with DNA and RNA. Energization of the ethidium-incubated cells led to a decrease in fluorescence. The mutant strains of *L. lactis* have an increased ethidium efflux relative to that of the wild-type strain (Fig. 3). The initial ethidium efflux rates for the Eth^R, Dau^R, and Rho^R strains were approximately seven-, five-, and threefold higher, respectively, than that for the wild-type strain. The difference in the stimulation of the efflux rate inferred from direct (Fig. 2) and indirect (Fig. 3) measurements is most likely due to the different ethidium concentrations at which transport was assayed. The differences in the ethidium efflux rates correspond to the differences in ethidium sensitivities of the various strains (Fig. 1). The data suggest that ethidium resistance is indeed caused by an active efflux process.

Ethidium efflux against a concentration gradient. If ethidium efflux is mediated by an energy-dependent transport process, efflux should occur against a concentration gradient. The free internal ($[Eth^{FREE}]_{IN}$) and external ($[Eth^{FREE}]_{OUT}$) ethidium concentrations were studied in the course of an efflux assay. The external free ethidium concentration ($[Eth^{FREE}]_{OUT}$) can be measured directly from the supernatant fraction of a cell suspension. The internal free ethidium concentration ($[Eth^{FREE}]_{IN}$) can be estimated from the fluorescence intensity of the polynucleotide-bound ethidium as described in Materials and Methods. The total fluorescence (F_{TOT}) was measured by incubating de-energized cells (treated with valinomycin and nigericin) for 3 h with different concentrations of ethidium bromide. From the calibration curve of Eth^R, a maximal fluorescence ($F_{BOUND\text{-}MAX}$) of 45.6 and a dissociation constant (K_D) of 1.05 μM were calculated. These values were used to estimate $[Eth^{FREE}]_{IN}$ and $[Eth^{FREE}]_{OUT}$ during the course of an efflux experiment (Fig. 4). Upon energization with glucose, $[Eth^{FREE}]_{IN}$ decreased from 1.7 to 0.12 μM, whereas $[Eth^{FREE}]_{OUT}$ increased from 1.7 to 4.4 μM, resulting in a 37-fold inwardly directed ethidium concentration gradient. In a comparable experiment with the wild-type strain, the steady-state $[Eth^{FREE}]_{IN}$ and $[Eth^{FREE}]_{OUT}$ were 0.75 and 3.75 μM, respectively, yielding a fivefold inwardly directed ethidium

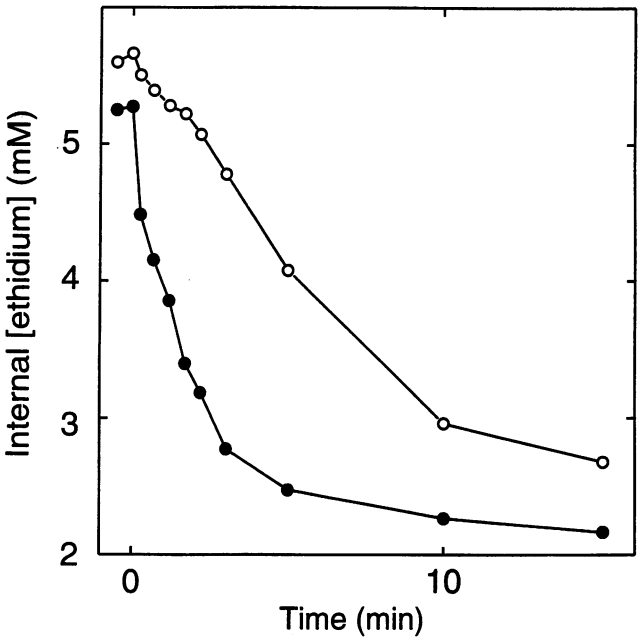

FIG. 2. Ethidium efflux from whole cells: direct measurement of ethidium concentration. Ethidium efflux from cells of *L. lactis* subsp. *lactis* MG1363 (wild type) (○) and Eth^R (●) preincubated for 2 h with 5 μM ethidium bromide was monitored after energization with glucose (25 mM). The internal ethidium concentration was calculated from the difference between the total amount added and the concentration measured in the supernatant fraction.

concentration gradient. These observations indicate that ethidium efflux is mediated by energy-dependent processes.

Inhibition by reserpine. Reserpine is known as a potent inhibitor of the MDR1 P-glycoprotein and of the *Bacillus subtilis* MDR carrier (4, 31). Cells were preincubated with reserpine (10 μg/ml) prior to the addition of ethidium. Reserpine inhibited ethidium efflux completely in the wild-type and Dau^R strains (Fig. 5A, RES) and Rho^R (data not shown) and partially in Eth^R (Fig. 6A, RES). Energization of *L. lactis* cells leads to the generation of an electrical potential ($\Delta\psi$; inside negative) which pulls the cationic ethidium to the inside, and the ethidium concentration gradient ($[Eth^{FREE}]_{IN}/[Eth^{FREE}]_{OUT}$) will become in equilibrium with the $\Delta\psi$. The enhanced increase in ethidium fluorescence upon inhibition of the drug efflux pump by reserpine as observed for the wild-type, Dau^R, and Rho^R strains (Fig. 5A; data not shown) is consistent with such a mechanism, i.e., passive equilibration of ethidium with the membrane potential. The absence of this phenomenon in Eth^R cells suggest that ethidium efflux in this strain is not completely inhibited by reserpine.

Presence of a secondary drug extrusion system. To establish the driving force for ethidium efflux in *L. lactis*, ionophores were used to selectively dissipate the components of Δp. Dissipation of the proton gradient (ΔpH) by the electroneutral proton/potassium antiporter nigericin or by the addition of the weak acid potassium acetate inhibited ethidium efflux in the wild-type and Dau^R strains (Fig. 5A, NIG) and in the Rho^R strain (data not shown). These results indicate that the ΔpH is a driving force for ethidium efflux. Addition of the potassium ionophore valinomycin enhanced the efflux of ethidium in all strains tested (Fig. 5A and 6A, VAL). Valinomycin dissipates the $\Delta\psi$, with a concomitant increase in the ΔpH (28). This decrease in the $\Delta\psi$ will lower the ethidium influx and/or stimulate the efflux irrespective of whether the Δp is directly involved in the efflux via the transport protein(s). Indications for the involvement of the $\Delta\psi$ in the ethidium efflux process were obtained from ethidium uptake experiments in cells in which the ethidium efflux pump was inactivated. Cell suspensions were energized with glucose and incubated at room temperature in the presence or absence of reserpine or nigericin. Uptake was initiated upon the addition of ethidium bromide (Fig. 5B). Ethidium uptake in reserpine-treated cells was higher than that in nigericin treated cells (ΔpH poised to zero), indicating that the $\Delta\psi$ is involved in ethidium efflux. Taken together, the results suggest that ethidium efflux in wild-type cells is mediated by a secondary transport system and that both components of Δp function as a driving force.

Presence of a primary drug extrusion system. In contrast to its effect on the wild-type, Dau^R, and Rho^R strains, nigericin had only a minor effect on ethidium efflux in Eth^R (Fig. 6A, NIG). Dissipation of Δp by protonophores [SF2487, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone (FCCP), and carbonyl cyanide *m*-chlorophenylhydrazone] or valinomycin plus weak acids like potassium acetate also did not significantly affect ethidium efflux in Eth^R (data not shown). These observations suggest a mechanism of energy coupling to ethidium efflux in Eth^R that is different from that in the wild-type, Dau^R, and Rho^R strains. To analyze whether ethidium efflux was coupled to ATP hydrolysis, the effects of various ATPase inhibitors on ethidium transport were examined. For this purpose, cells were preenergized with 10 mM L-arginine and preincubated with different ATPase inhibitors. Assays were started by the addition of 10 μM ethidium bromide. *ortho*-Vanadate (0.5 mM) increased the level of ethidium uptake,

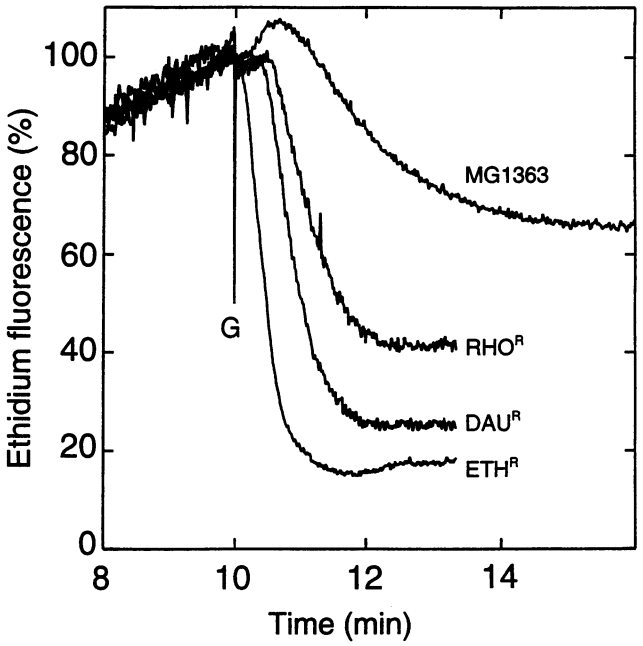

FIG. 3. Ethidium efflux from whole cells: indirect measurement of ethidium concentration. Ethidium at a final concentration of 10 μM was added to cell suspensions of *L. lactis* subsp. *lactis* MG1363 (wild type), Eth^R, Dau^R, and Rho^R. After 10 min of equilibration, the cells were energized by adding 25 mM glucose, and ethidium efflux was monitored by measuring the fluorescence decrease. The fluorescence intensity after the addition of ethidium and before the addition of the energy source was normalized to 100%.

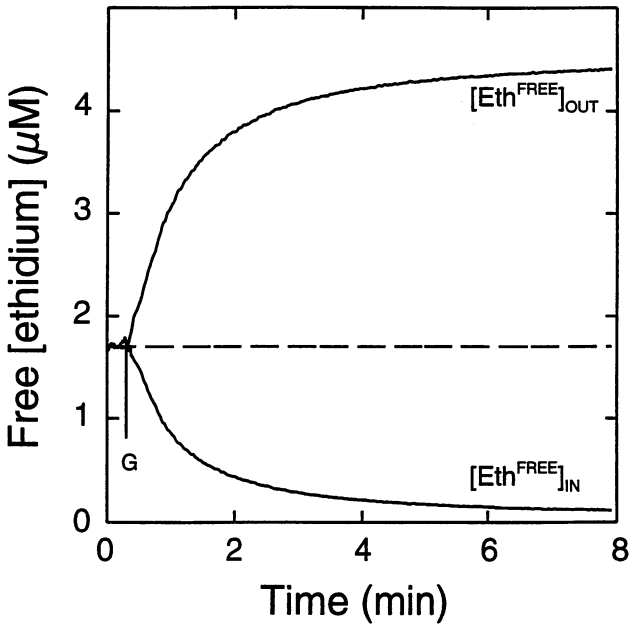

FIG. 4. Ethidium efflux against a concentration gradient. Deoxyglucose-treated cells of *L. lactis* subsp. *lactis* Eth^R were incubated with ethidium (5 μM) in the presence of valinomycin (1 μM) plus nigericin (1 μM) until a steady state was reached ($[Eth^{FREE}]_{IN} = [Eth^{FREE}]_{OUT} = 1.70$ μM). $[Eth^{FREE}]_{IN}$ and $[Eth^{FREE}]_{OUT}$ after addition of glucose (25 mM) were determined as described in Materials and Methods.

indicating that ethidium efflux was inhibited (Fig. 6B). Arsenate (0.5 mM) and *N,N'*-dicyclohexylcarbodiimide (0.5 mM) inhibited ethidium efflux only partially, while sodium azide (0.5 mM) had no effect (data not shown). *ortho*-Vanadate inhibited ethidium efflux in Dau^R and Rho^R partially but did not affect ethidium efflux from the wild-type strain (data not shown). Since arginine metabolism via the arginine deiminase (ADI) pathway and the concomitant synthesis of ATP are not affected by *ortho*-vanadate, these observations indicate that the efflux system is ATP driven. Taken as a whole, the experiments with the metabolic inhibitors indicate that *L. lactis* is able to express at least two different (ethidium) efflux activities. One activity is present in the wild-type strain and forms the major system for ethidium efflux in Dau^R and Rho^R. This system is coupled to Δp. The second system is most likely coupled to ATP and is responsible for ethidium efflux in Eth^R. This system appears to play a minor role in the efflux from Dau^R and Rho^R strains.

Daunomycin is a substrate of the *L. lactis* MDR transporters. Daunomycin fluorescence is quenched upon binding to polynucleotides, and this property can be used to determine the internal daunomycin concentration. Efflux of this probe results in an increase in the fluorescence signal (38). The initial rate of daunomycin efflux from wild-type cells was increased upon energization with glucose (25 mM). The daunomycin efflux rate in Eth^R was much higher than that in the wild-type strain (Fig. 7). Similar results were obtained with Dau^R and Rho^R strains (data not shown). Moreover, as observed for ethidium efflux, daunomycin efflux was sensitive to valinomycin and nigericin in wild-type cells but not in Eth^R cells. These data indicate that daunomycin is also a substrate of the lactococcal MDR transporters and that an increased resistance

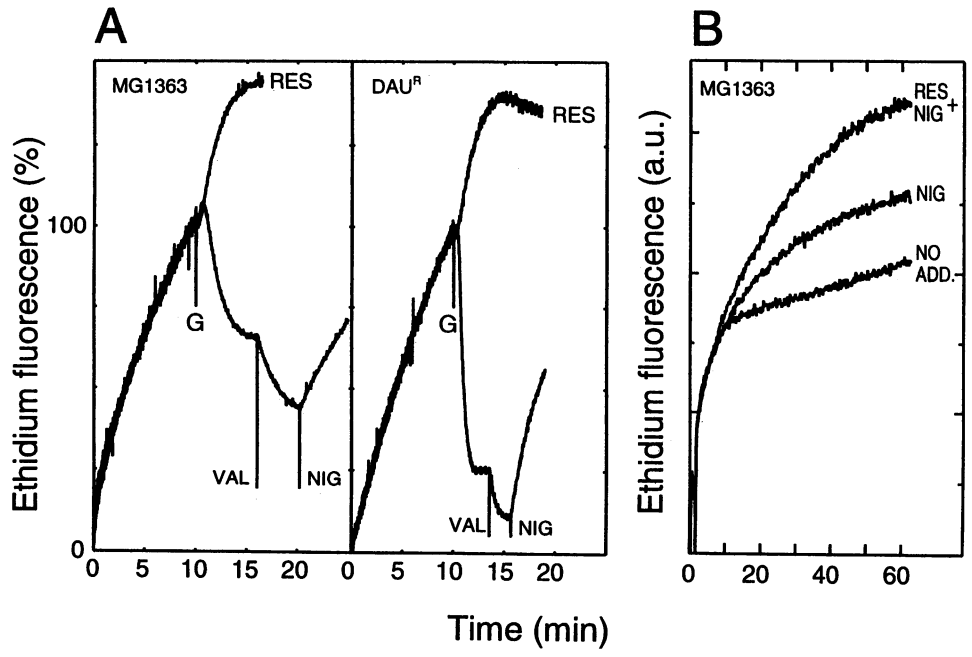

FIG. 5. Effect of ionophores and reserpine on ethidium efflux and uptake in *L. lactis* subsp. *lactis* MG1363 (wild type) and Dau^R. (A) MG1363 and Dau^R cells (0.2 mg of protein per ml) were incubated with 10 μM ethidium and subsequently energized with 25 mM glucose (G). Valinomycin (VAL) and nigericin (NIG) were added to a final concentration of 1 μM (lower curve) as indicated. Reserpine (RES) (10-μg/ml final concentration) was added prior to incubation with ethidium. The fluorescence intensity after the addition of ethidium and before the addition of the energy source was normalized to 100%. (B) Uptake of ethidium (1.25 μM) in preenergized MG1363 cells (0.1 mg of protein per ml) (NO ADD.) and in cells preincubated in the presence of nigericin (NIG) or reserpine plus nigericin (RES + NIG) at the same concentrations as for panel A.

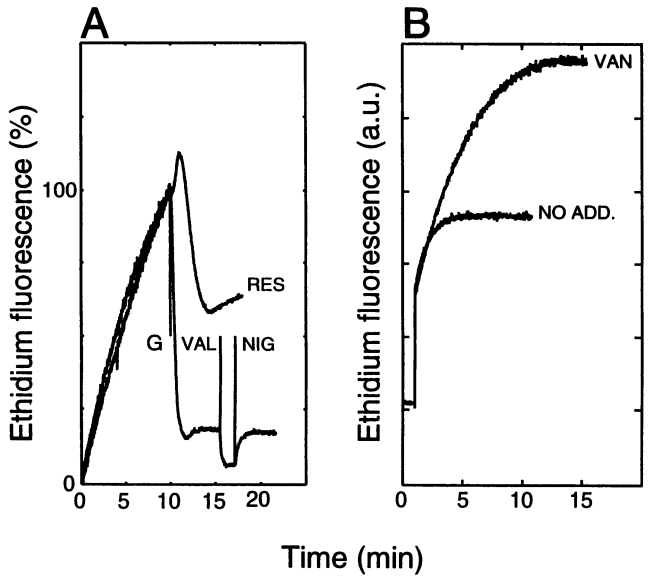

FIG. 6. Effect of ionophores, reserpine, and *ortho*-vanadate on ethidium efflux and uptake in *L. lactis* subsp. *lactis* Eth^R. (A) Eth^R cells (0.2 mg of protein per ml) were incubated with 10 μM ethidium and subsequently energized with 25 mM glucose (G). Valinomycin (VAL) and nigericin (NIG) were added to a final concentration of 1 μM (lower curve) as indicated. Reserpine (RES) (10-μg/ml final concentration) was added prior to incubation with ethidium. The fluorescence intensity after the addition of ethidium and before the addition of the energy source was normalized to 100%. (B) Eth^R cells were preincubated for 10 min in a phosphate-free buffer (potassium-HEPES [pH 7.0] plus 5 mM $MgSO_4$) supplemented with 0.5 mM *ortho*-vanadate (VAN) and were preenergized with L-arginine (10 mM) to allow *ortho*-vanadate to enter the cells. The uptake experiment was started upon the addition of 10 μM ethidium. The lower curve represents ethidium uptake in the absence of *ortho*-vanadate.

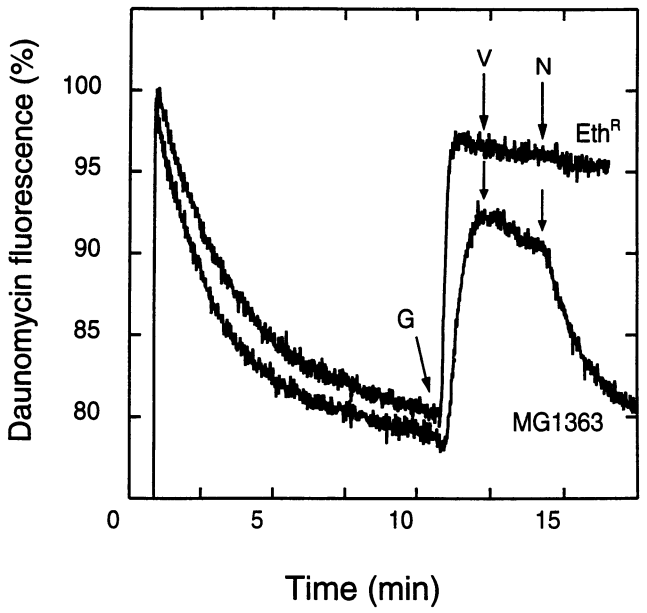

FIG. 7. Daunomycin efflux in *L. lactis* subsp. *lactis* MG1363 (wild-type) and Eth^R. Daunomycin was added to cell suspensions (0.2 mg of protein per ml) of *L. lactis* subsp. *lactis* MG1363 (wild type) and Eth^R to a final concentration of 10 μM. Passive influx of daunomycin into the cells was monitored by measuring the fluorescence decrease (excitation at 480 nm and emission at 590 nm) as a result of binding of daunomycin to polynucleotides. After 10 min, the efflux experiment was started by the adding glucose (G) (25 mM). The maximal fluorescence intensity immediately following the addition of daunomycin was normalized to 100%. V, valinomycin; N, nigericin.

towards daunomycin can be at least partially explained by increased extrusion of the drug.

DISCUSSION

Mutants of *L. lactis* subsp. *lactis* MG1363, termed Eth^R, Dau^R, and Rho^R, which display an MDR phenotype have been isolated and characterized. The mutants were isolated by selecting for resistance to three structurally unrelated toxic compounds, i.e., ethidium bromide, daunomycin, and rhodamine 6G. All three mutants showed a similar pattern of cross-resistance to a variety of unrelated drugs, among which were typical substrates for the human P-glycoprotein. Analogous to MDR in mammalian cells and different bacteria, the MDR phenotype in *L. lactis* is associated with active drug extrusion. This conclusion is based on measurements of both the internal and external drug concentrations with fluorometric assays. Drug efflux in the three MDR mutants is an energy-dependent enzyme-mediated process as demonstrated by (i) the stimulation of drug efflux upon energization with glucose (each mutant exhibited an energy-dependent efflux of both ethidium and daunomycin which was higher than that in the wild-type strain) and (ii) drug efflux against a concentration gradient. The increased resistance of the mutants to ethidium and daunomycin must be at least partially due to an increase in extrusion of these compounds. It is likely that the increased drug extrusion is also responsible for the cross-resistance to the other compounds tested (e.g., rhodamine 6G, actinomycin D, gramicidin D, and quinine). Increased drug efflux in the different mutants might be the result of an increased expression of the systems and/or an increased catalytic efficiency in transporting the drugs.

Analysis of the energy coupling to ethidium efflux indicates that in *L. lactis* more than one transport system can be involved in the efflux process. Although it cannot be excluded that the peptide ionophore nigericin (and perhaps valinomycin) is a substrate of the lactococcal drug efflux system, the observation that dissipation of the ΔpH by nigericin and weak acids (e.g., acetate) abolishes essentially all efflux activity strongly suggests that this process is coupled to the ΔpH. Since dissipation of the ΔpH component of the Δp at pH 6.5 is not sufficient to block ethidium efflux from the wild-type strain completely, an involvement of the $\Delta\psi$ in the energy coupling appears most likely. These observations are best explained by an electrogenic ethidium/proton antiport system that couples the efflux of ethidium (carrying one positive charge) to the uptake of at least two protons. Ethidium extrusion from Eth^R cells is inhibited by the phosphate analog *ortho*-vanadate and not by nigericin, protonophores, or weak acids that dissipate the Δp or components thereof. This is indicative of an ATP-dependent transport system. Reserpine, a plant alkaloid which inhibits the transport catalyzed by the vesicular amine transporter (1), P-glycoprotein (4, 7), and the *B. subtilis* MDR carrier (1), also inhibits ethidium efflux in wild-type *L. lactis* and in the Dau^R, Rho^R, and Eth^R strains. Apparently, both the Δp-driven and the ATP-dependent systems are inhibited by reserpine, although inhibition of the latter system is less pronounced than that of the Δp-dependent system. It is worth noting that drug efflux occurs against a concentration gradient

in both the wild-type and Eth^R strains. To dissect the putative Δp-driven and ATP-dependent drug efflux activities in *L. lactis*, a genetic approach is currently being pursued.

The pronounced extrusion activity in the Eth^R mutant is not mediated by the previously described ATP-driven 2′,7′-bis-(2-carboxyethyl)-5[and-6]-carboxyfluorescein (BCECF) efflux system of *L. lactis* subsp. *lactis* ML3 (30). This conclusion is based on the observations that the rate of ethidium efflux is not changed in a mutant which has a fourfold-lowered rate of BCECF efflux and that the rate of BCECF efflux in the Eth^R strain is the same as that in the wild-type (29).

The presence of an ATP-dependent transport system involved in MDR in prokaryotes has not been described before. The ATP-dependent efflux systems which have been described for prokaryotes extrude or confer resistance to only a small range of related compounds (recently reviewed in references 5 and 12). Furthermore, the similarity of these systems to P-glycoprotein is restricted to highly conserved regions around the nucleotide-binding motifs (10, 34–36) which bear no direct relationship to a MDR phenotype. Although the gene(s) encoding the ATP-dependent ethidium efflux system has not yet been isolated and a structural similarity cannot be established, the lactococcal system has a number of functional properties in common with P-glycoprotein; i.e., both systems facilitate the efflux of a wide variety of unrelated drugs and are inhibited by *ortho*-vanadate and reserpine.

Multidrug efflux systems in both gram-positive and gram-negative organisms that are driven by the Δp have been described previously (23, 31, 39, 42). These energy-dependent efflux systems are believed to be drug/proton antiporters (17, 23, 31, 41), although solid evidence for such a mechanism is lacking. The physiological function of the ethidium extrusion system(s) could be related to a need for the organism to extrude toxic compounds encountered in the natural habitat, such as toxins produced by microorganisms or plants. Many of these compounds are both water and lipid soluble (7, 13).

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